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## Molecular tracking of antigen-specific T cell clones in neurological immune-mediated disorders

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### Summary

T cells recognizing self or microbial antigens may trigger or reactivate immune-mediated diseases. Monitoring the frequency of specific T cell clonotypes to assess a possible link with the course of disease has been a difficult task with currently available technology. Our goal was to track individual candidate pathogenic T cell clones, selected on the basis of previous extensive studies from patients with immune-mediated disorders of the CNS, including multiple sclerosis, HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/ TSP) and chronic Lyme neuroborreliosis. We developed and applied a highly specific and sensitive technique to track single CD4<sup>+</sup> and CD8<sup>+</sup> T cell clones through the detection and quantification of T cell receptor (TCR)  $\alpha$  or  $\beta$  chain complementarity-determining region 3 transcripts by real-time reverse transcriptase (RT)-PCR. We examined the frequency of the candidate pathogenic T cell clones in the peripheral blood and CSF during the course of neurological disease. Using this approach, we detected variations of clonal frequencies that appeared to be related to clinical course, significant enrichment in the CSF, or both. By integrating clono-type tracking with direct visualization of antigen-specific staining, we showed that a single T cell clone contributed substantially to the overall recognition of the viral peptide/MHC complex in a patient with HAM/ TSP. T cell clonotype tracking is a powerful new technology enabling further elucidation of the dynamics of expansion of autoreactive or pathogen-specific T cells that mediate pathological or protective immune responses in neurological disorders.

### Keywords

autoimmunity; HAM/TSP; Lyme disease; multiple sclerosis; T cell receptors; immunotherapy

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## Introduction

The activation and expansion of antigen-specific T cells are considered crucial events for the initiation of organ-specific inflammatory disorders. A possible role of pathogenic T cell responses has been extensively investigated in inflammatory diseases of the CNS such as multiple sclerosis, HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/ TSP) and chronic Lyme neuroborreliosis. Multiple sclerosis, the most frequent demyelinating disease of the CNS, is characterized by focal areas of inflammation and loss of myelin in the white matter of the brain and spinal cord, together with variable extent of axonal damage, atrophy and gliosis. The most common clinical course, particularly early after onset, is relapsing-remitting (RRMS). While the causative agent of multiple sclerosis is unknown, myelin antigen-specific CD4<sup>+</sup> T cells are thought to play a role in the inflammatory relapses that lead to myelin destruction (Martin and McFarland, 1995; Steinman, 1996; Hohlfeld and Wekerle, 2001). Myelin basic protein (MBP) has been implicated as one of the antigens recognized by pathogenic T cells (Hafler and Weiner, 1995; Bielekova *et al.*, 2000). HAM/TSP is a slowly progressive neurological syndrome characterized by paraparesis, sensory and sphincter dysfunction. The main histopathological abnormalities are thoracic spinal cord atrophy with perivascular demyelination and axonal degeneration. In HAM/TSP, the high frequency of HTLV-I *Tax*-specific cytotoxic T lymphocytes (CTL) in the patients' peripheral blood and CSF (Jacobson *et al.*, 1990; Elovaara *et al.*, 1993; Greten *et al.*, 1998; Bieganowska *et al.*, 1999), their oligoclonal expansion (Utz *et al.*, 1996) and their proinflammatory phenotype (Biddison *et al.*, 1997; Kubota *et al.*, 1998; Bieganowska *et al.*, 1999) have suggested a retrovirus-induced T-cell mediated pathogenesis. Chronic Lyme neuroborreliosis or post-treatment Lyme disease syndrome probably represents an immune-mediated organ manifestation that is triggered by *Borrelia burgdorferi* in genetically susceptible individuals. Our group has recently examined the antigenic recognition of CSF-derived T cells that responded to *B. burgdorferi* proteins and to self-antigens in a patient with chronic Lyme neuroborreliosis (Hemmer *et al.*, 1999). These findings suggested that molecular mimicry (Wucherpfennig and Strominger, 1995) may be one of the mechanisms leading to autoimmunity in patients suffering from this disorder.

Similar to other organ-specific immune-mediated disorders, in these neurological diseases current data suggest a pathogenic role of antigen-specific T cells in the inflammatory process. However, mainly due to technical difficulties, important questions remained elusive so far. In particular, it has been problematical to follow the frequency of candidate pathogenic T cell clones over time, particularly in target organ compartments, and to assess a potential relationship of an individual clone with the course of disease. Here, we hypothesized that the different clinical, pathological and immunological features of these neurological disorders may be associated with different profiles of clonal dynamics or distribution. The alternating phases of RRMS were an appropriate context to assess a possible relationship between longitudinal variation of clonal frequency and markers of disease activity, such as clinical exacerbations or the detection of active lesions by MRI. In contrast, HAM-TSP may be considered a prototypic example of a chronic-progressive immunemediated CNS disorder initiated and sustained by a persistent viral infection. Chronic Lyme neuroborreliosis may be seen as a paradigm for a chronic-relapsing disorder

in which immune-mediated inflammatory damage can continue even after the causal infectious agent has been cleared from the body. To address these questions, we have developed and applied a novel technology termed T cell clonotype tracking (TCCT) to follow single CD4<sup>+</sup> and CD8<sup>+</sup> T cell clones in a highly specific and sensitive manner.

In principle, we quantified TCR transcripts that originate from a single T cell clone by real-time reverse transcriptase (RT)-PCR using oligonucleotide primers that flank the clones' TCR complementarity-determining region 3 (CDR3). Using this approach we tracked T cell clones for which the results of previous extensive studies suggested a pathogenic involvement, based on their recognition of immunodominant epitopes of disease-related antigens, their clonal expansion, and their phenotypic and functional characteristics. In a patient with multiple sclerosis we tracked T cell clone P2-10, a CD4<sup>+</sup> T helper 1 (Th1) clone that recognized the central immunodominant epitope of MBP, MBP<sub>83-99</sub> (Bielekova *et al.*, 2000). In a patient with HAM/ TSP we tracked A6, a cytotoxic CD8<sup>+</sup> T cell clone which recognized the immunodominant HLA-A2-restricted HTLV-I *Tax* epitope *Tax*<sub>11-19</sub> (Utz *et al.*, 1996). In a patient with chronic Lyme neuroborreliosis we tracked CSF3, a CD4<sup>+</sup> Th1 clone that recognized multiple pathogen-derived and self-antigen epitopes (Hemmer *et al.*, 1999). As control we used a MBP<sub>111-129</sub>-specific, superantigen-responsive T cell clone (HD4-1C2) that was found clonally expanded in a healthy subject, and has also been studied comprehensively (Muraro *et al.*, 1997). We determined the frequencies of the selected T cell clones in the peripheral blood compartment and in available CSF cell specimens and examined their relationship with clinical aspects of disease. The results illustrate the power of our new methodology and reveal important features of the kinetics of frequency and of the distribution of the candidate pathogenic T cell clones during the course of neurological disease.

## Material and methods

### Case reports

Patient MS502 is a 31-year-old Caucasian male diagnosed with clinically definite RRMS according to Poser's criteria (Poser *et al.*, 1983) 10 years ago. He has active disease by both clinical and MRI criteria, with predominance of corticospinal motor and brainstem dysfunction. His treatment history includes 15 months of cyclosporin (1991-1992; good response on MRI inflammatory activity), 18 months of interferon  $\beta$ -1b (1994-1995; very good clinical and MRI response, discontinued because of local reactions and depression), 5 weeks of altered peptide ligand (APL) CGP77116 based on MBP<sub>83-99</sub> sequence (1998; discontinued because of treatment-related atypical multiple sclerosis exacerbation) (Bielekova *et al.*, 2000) and since 1999 treatment with interferon  $\beta$ -1a, resulting in good clinical and MRI response with current expanded disability status scale (EDSS; Kurtzke, 1983) score 1.5.

Patient HAM3 is a 69-year-old Caucasian male diagnosed with HAM-TSP 12 years ago, based on clinical presentation, MRI findings and HTLV-I positive serology. However, the characteristic clinical symptoms (persistent back pain, leg weakness, urinary problems) started 33 years ago. His treatment history consisted of Anti-TAC (Zenapax, humanized

mAb against CD25) for 4 months in 1995, without significant clinical response. His clinical course showed continuous slow progression of disability, with a current EDSS 6.5.

Patient NB1 is a 34-year-old Caucasian male who presented in 1993 with meningoencephalitis and cerebral vasculitis. He was diagnosed with Lyme borreliosis based on the positive serological results, according to Centers for Disease Control criteria and high anti-borrelial intrathecal antibody levels (CSF/serum index: 2.05). His neurological symptoms resolved upon antibiotic therapy (ceftriaxone 2 g intravenously per day for 4 weeks), but recurred multiple times in the subsequent 7 years. He has persistent high titres of *B. burgdorferi*-specific antibodies in serum and CSF and positive intrathecal antibody index. CSF was repeatedly negative for *B. burgdorferi* by both PCR and culture of the organism.

Healthy blood donor HD4, a 35-year-old Afro-American male, was enrolled at the NIH Blood Bank.

All patients were followed under research protocols reviewed and approved by NIH Institutional Review Boards and written informed consent was obtained from all subjects in conformity to the Declaration of Helsinki.

### T cell clones

The MBP<sub>83-99</sub>-specific CD4<sup>+</sup> T cell clone P2-10 was isolated from peptide-stimulated peripheral blood mononuclear cells (PBMC) from a patient with multiple sclerosis (MS502) after a screening of peripheral blood T cell responses employing a panel of 15 myelin protein-derived immunodominant peptides in an interleukin (IL)-7-modified proliferation assay, as described previously (Bielekova *et al.*, 1999, 2000). The MBP<sub>111-129</sub>-specific CD4<sup>+</sup> T cell clone 1C2 was established from MBP-stimulated peripheral blood mononuclear cells (PBMC) from a healthy donor (HD4) by split-well cloning as described previously (Muraro *et al.*, 1997). Clone 1C2 responds to staphylococcal enterotoxin A (SEA) at low nanomolar concentrations of the superantigen (P. A. Muraro, unpublished data). The HTLV-I Tax<sub>11-19</sub>-specific CD8<sup>+</sup> T cell clone A6 was generated from CD8<sup>+</sup> cells from a patient with HAM/TSP (HAM3) stimulated with peptide-pulsed PBMC as described previously (Utz *et al.*, 1996). The *B. burgdorferi*-specific CD4<sup>+</sup> T cell clone CSF3 was cloned by limiting dilution from CSF cells of patient NB1 stimulated with *B. burgdorferi* lysate as described previously (Hemmer *et al.*, 1999). The phenotypic characteristics of T cell clones are summarized in Table 1. Expansion of the selected T cell clones in the peripheral blood or in the CSF of individual patients has been shown by limiting dilution analysis followed by TCR sequencing or single-strand conformational polymorphism (Utz *et al.*, 1996; Muraro *et al.*, 1997; Hemmer *et al.*, 1999; Bielekova *et al.*, 2000).

### TCR analysis and sequencing

An analysis of T cell clones TCR V gene usage was performed by PCR using a panel of 21 TCRAV or 22 TCRB V family-specific oligonucleotide primers (Utz *et al.*, 1996). Nucleotide sequencing of TCR rearrangements was performed by either manual or automated sequencing as described previously (Muraro *et al.*, 1997, 2000). Expression of functional TCR rearrangements on the cell surface was confirmed by FACS staining employing a panel of anti-TCRBV monoclonal antibodies as described previously (Muraro

*et al.*, 2000). TCR designations are given according to the WHO guidelines (WHO IUIS Nomenclature Sub-Committee on TCR Designation, 1995) following Arden's nomenclature (Arden *et al.*, 1995).

### Molecular detection and quantification of T cell clonal frequency

Total RNA was isolated from clonal cells, PBMC and CSF cells using TriZol reagent (Gibco-BRL, Gaithersburg, MD, USA). RNA was reverse transcribed into cDNA using random hexamer primers and TaqMan Reverse Transcription Reagents (PE Biosystems, Foster City, CA, USA). Oligonucleotide primers and probes were designed using Primer Express software (PE Biosystems) according to criteria that aimed to obtain amplification of clonotypic sequences with maximum specificity. The most efficient primers ranged from 18 to 24 nucleotides. Probes were chosen to be 22–29 nucleotides long to achieve maximum specificity. Resulting amplicons ranged in size from 75 to 100 bp.

We excluded significant homologies between CDR3 rearrangements and genomic sequences by running extensive searches on the GenBank database. The annealing sites of selected clonotype-specific primer/probe combinations are shown in Fig. 1. In preliminary experiments we ruled out non-target amplification from control genomic DNA samples and confirmed the specific amplification of target TCR rearrangements by DNA electrophoresis and direct automated sequencing of PCR amplicons. For all experiments, real-time quantitative RT-PCR was performed on an ABI Prism 7700 Sequence Detection System (PE Biosystems) using the TaqMan Gold RT-PCR Kit (PE Biosystems) according to the manufacturer's recommendations. Briefly, this method monitors the degradation of a dual-labelled fluorescent probe (TaqMan probe) in real time concomitant with PCR amplification (Heid *et al.*, 1996). Input target RNA levels are correlated with the time (measured in PCR cycles) at which the reporter fluorescent emission increases beyond a threshold level. PCR conditions were: one cycle at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Oligonucleotides (PE Biosystems) were used at final concentrations of 200 nM for forward and reverse primer and 100 nM for the fluorogenic probe. We first assessed the specificity of each PCR primer/probe combination against cDNA from the target T cell clone and controls including 12 non-target clones and PBMC from six unrelated subjects. Selected primer/probe sets led to efficient amplification of target cDNA equivalent to  $5 \times 10^3$  cells/reaction, with mean threshold cycle ( $C_T$ ) values ranging from 7 to 21 cycles (Table 2). There was some non-specific amplification of non-target cDNA from some allogeneic PBMC samples (equivalent to  $10^5$  cells/reaction) only above 31–35 PCR cycles. Thus, a difference of mean  $C_T$  number from amplification of non-target and target T cell ( $\Delta C_T$ ) of a minimum of 10 PCR cycles (corresponding to a >1000-fold difference in amplification product) provided a robust margin of security for a selective detection of target sequences even in presence of a large excess of non-target cells. To assess the sensitivity of TCCT, we 'spiked' different numbers of clonal T cells into constant numbers of allogeneic PBMC. T cell clones employed in spiking experiments were in a resting state (>12 days after last restimulation) to minimize the potential increase of number of TCR transcripts per individual T cell induced by recent *in vitro* activation (Kurokawa *et al.*, 1999; Nixon *et al.*, 1999). Compared with clonotype tracking employing conventional quantitative end-point PCR (Fig. 2A), TaqMan PCR was

on average 10-fold more sensitive, resulting in a lower limit of detection of 1 target cell/10<sup>6</sup> control cells for clone 1C2 (Fig. 2B), 1 cell/10<sup>6</sup> for T cell clone P2–10, 1 cell/5 × 10<sup>4</sup> for T cell clone A6 and 1 cell/ 5 × 10<sup>6</sup> for T cell clone CSF3 (Fig. 2C). Relative quantification of gene expression was calculated using the C<sub>T</sub> method (Weiner *et al.*, 1999). Amplification of GAPDH or 18S rRNA (for *in vitro* stimulated cell cultures) was used for sample normalization as described previously (Wandinger *et al.*, 2001). To determine the absolute frequencies of target cells, 10<sup>0</sup>-10<sup>6</sup> clonal cells were spiked at 10-fold increment into either 10<sup>6</sup> or 5 × 10<sup>6</sup> allogeneic PBMC. The normalized PCR amplification signals derived from the cDNAs served as calibrators. Then, each of the normalized target values for the experimental samples was divided by the normalized value of the calibrator. All reactions were run in duplicate and results were always confirmed by at least one additional independent run.

We next attempted to detect the expansion of clonal cells after *in vitro* stimulation of the ‘parent’ PBMC from which a clone had originally been established. For this purpose, we tracked the frequency of the MBP<sub>111–129</sub>-specific, SEA-responsive T cell clone 1C2 upon primary stimulation of the unmanipulated parent PBMC from donor HD4 with antigens and superantigens (Fig. 3). After 72 h incubation at 37° C, an expansion of the T cell clone was detected in cDNA from the PBMC cultures stimulated with MBP<sub>111–129</sub> and SEA (Toxin Technologies, Sarasota, FL, USA), but not from those stimulated with the control antigen Flu-HA<sub>307–319</sub>, the control superantigen staphylococcal enterotoxin E (SEE) or IL-2 alone. The reliable detection of specifically activated T cells emerging from a bulk PBMC population after *in vitro* stimulation confirmed the potential of our approach for tracking T cell expansions *ex vivo*.

### FACS staining of antigen-specific cells with Tax<sub>11–19</sub>-A2/Ig

To enumerate Tax<sub>11–19</sub>-specific, A2-restricted T cells, we used a soluble Tax<sub>11–19</sub>/HLA-A2/Ig fusion protein for FACS staining as described previously (Greten *et al.*, 1998). To check the specificity of Tax<sub>11–19</sub>-A2/Ig recognition, we used as control the same A2/Ig construct loaded with an HIV peptide (HIV gag<sub>77–85</sub>).

## Results

### Tracking of P2–10, a CD4<sup>+</sup> T cell clone specific for the immunodominant peptide MBP<sub>83–99</sub>, in a patient with multiple sclerosis

A clinical trial with an altered peptide ligand (APL) based on the immunodominant MBP<sub>83–99</sub> peptide as a novel treatment for multiple sclerosis (Bielekova *et al.*, 2000) gave us the opportunity to track the frequency of a potentially pathogenic T cell clone in multiple sclerosis. Five weeks after the start of APL treatment, patient MS502 presented with symptoms of a severe clinical relapse. MRI of the brain showed an impressive bout of 91 contrast-enhancing lesions (Fig. 4A, month 1.25). APL treatment was halted and intravenous steroid treatment was given, resulting in clinical and MRI remission. The patient suffered a second, milder clinical relapse 5 months after the start of APL treatment. The peripheral blood T cell response to myelin antigens was examined at two points in time by proliferation assay using a panel of 15 myelin protein-derived immunodominant peptides. One week

before the onset of the first exacerbation, MBP<sub>83-99</sub>-specific T cells were found expanded in the peripheral blood (Fig. 4B). While reactivity to MBP<sub>83-99</sub> as assessed by primary proliferation was strikingly predominant in the first relapse, the response to this epitope was markedly reduced at the time of the second relapse. In contrast, proteolipid protein (PLP)<sub>190-209</sub>-specific T cell responses increased at the time of the second relapse. The MBP<sub>83-99</sub>-specific T cell clone P2-10, isolated at the time preceding the first relapse, responded to extremely low concentrations (0.1 pg/ml) of native antigen, cross-reacted with the APL only at high doses and had a Th1 phenotype (Bielekova *et al.*, 2000; Table 1 and data not shown). These functional characteristics strongly suggested a pathogenic relevance of MBP<sub>83-99</sub>-specific T cell clone P2-10. We next assessed the possible correlation of its frequency with the course of disease. Employing TCCT, we found a >5-fold increase in the frequency of P2-10, 1 week prior to the first clinical exacerbation and the observed flare of MRI disease activity (Fig. 4C). After clinical remission, the frequency of the clone decreased to baseline levels. T cell clone P2-10 was not found expanded during the second exacerbation, which was associated with an increased cellular immune reactivity to PLP and not to MBP. The absolute frequency of P2-10 in PBMC at 1 week before the first exacerbation during the clinical trial was 5.5 cells/10<sup>4</sup> (or, 1 cell in 1818). Of note, we also detected the clone in the patient's peripheral blood over 6 years before the APL trial, at a frequency of 2.4 P2-10 cells/10<sup>4</sup> (or, 1 cell in 4167) at the time of an exacerbation, a value above those observed during disease remission (1.3–1.6 P2-10 cells/10<sup>4</sup>). As control, we monitored in healthy donor HD4 the circulating frequency of the MBP<sub>111-129</sub>-specific control T cell clone 1C2. Unlike the MBP-specific clone in the multiple sclerosis patient, clonal frequency of 1C2 in the peripheral blood of donor HD4 remained very stable with only minor fluctuations during a period of >4 years (data not shown). The large variation of clonal frequency in the patient with RRMS and the stability in the healthy donor appear to recapitulate at the level of single T cell clones the results of a serial study of whole peripheral blood TCR BV gene expression, which showed that skewed TCR repertoires associated to oligoclonal T cell expansions are frequently found in multiple sclerosis patients and not in healthy controls (Muraro *et al.*, 2002).

### Tracking of A6, a T cell clone specific for the immunodominant peptide HTLV-I Tax<sub>11-19</sub>, in a patient with HAM/TSP

We determined the absolute frequency of the HTLV-I Tax<sub>11-19</sub>-specific CD8<sup>+</sup> T cell clone A6 in PBMC, CD8<sup>+</sup> subsets and CSF from patient HAM3. The frequency of the clone in PBMC, measured at five time points spanning a period of 7 years, was high and remarkably stable, ranging from 0.95 to 1.3 A6 cells/10<sup>3</sup> PBMC (mean 1.17/10<sup>3</sup>, or 1 out of 850 PBMC; Fig. 5A). As expected, the clone was enriched in the CD8<sup>+</sup> subset, with a frequency of 23.9/10<sup>3</sup> at a first time point (1996) and 21.1/10<sup>3</sup> at a second time point (1997-1), representing on average 22.5/10<sup>3</sup>, or 1 out of 44 CD8<sup>+</sup> cells (Fig. 5B). The frequency of A6 in the CSF was 15.3/10<sup>3</sup> cells (1 out of 65 cells), a striking ~12-fold enrichment with respect to the corresponding peripheral blood (1.3 A6 cells/10<sup>3</sup> PBMC; Fig. 5B). We next examined the relationship of A6 frequency with the overall recognition of the Tax epitope/A2 complex in the peripheral blood. The recognition of Tax<sub>11-19</sub>/A2 Ig at three time-points was also stable, resulting in a staining of 15.02–16.45% of CD8<sup>+</sup> T cells in PBMC (mean 15.85%, or 1 out of 6.3 CD8<sup>+</sup> cells; Fig. 5C). By combining FACS staining of HTLV-I Tax/A2-specific

CD8<sup>+</sup> cells and molecular clonotype tracking, we calculated that clone A6 alone represents 14.2%, or one out of seven, of all *Tax*<sub>11-19</sub>-specific, HLA-A2-restricted CD8<sup>+</sup> T cells in the peripheral blood (Fig. 5D).

### Tracking of CSF3, a *B. burgdorferi*-specific T cell clone derived from the CSF of a patient with chronic Lyme neuroborreliosis

We followed the frequency of T cell clone CSF3, a clone isolated from the CSF of patient NB1, in seven PBMC and three CSF samples collected over a period of 2.5 years. The frequency of CSF3 was below our lower limit of detection of 1 cell/ $5 \times 10^6$  (Fig. 2C) for all PBMC samples tested. In contrast, we repeatedly detected CSF3 at a relatively high frequency in the CSF (Fig. 6). The absolute frequency of T cell clone CSF3 in the CSF during a severe clinical exacerbation (stroke-like manifestation involving the left basal ganglia and internal capsule) was increased 14-fold ( $25.3/10^4$ , or 1 CSF3 cell per 395 cells) compared with the frequency found during a recovery phase (1.8 CSF3 cells/ $10^4$ ). An increase of 2.8-fold compared with the frequency during remission occurred during a mild clinical exacerbation (mild persistent headache, with gadolinium-enhancement of an old right caudate lesion on MRI, and normal CSF cell count and protein levels).

## Discussion

Current evidence from studies in humans and in experimental animal models suggests that the broader T cell repertoire found following immune destruction of CNS tissue may represent the intermolecular dispersion of an initially focused, oligoclonal immune response (Lehmann *et al.*, 1992; McRae *et al.*, 1995; Steinman, 1996; Tuohy *et al.*, 1997). In addition to triggering the initial immune response, minor populations of lymphocytes may play an important role in defining the immune microenvironment within established lesions (Brosnan and Raine, 1996). Limited clonal heterogeneity has been shown in lymphocytes infiltrating multiple sclerosis plaques (Oksenberg *et al.*, 1990; Babbe *et al.*, 2000). Here, we applied a highly specific approach to assess the frequency of selected individual T cell clonotypes in three CNS disorders with immune involvement and different causes. Indeed, our goal was not to characterize the diversity of the pathogenic effector T cell repertoire in these diseases, but rather to probe the dynamics of frequency of immunodominant autoreactive and foreign Ag-specific T cell clones that were considered representative of potentially pathogenic responses based on previous extensive studies. Rather than a primary clonotyping technique such as CDR3 spectratyping (Immunoscope), which allows the detection of clonal expansions through the analysis of length distribution in TCR transcripts (Cochet *et al.*, 1992; Babbe *et al.*, 2000; Goebels *et al.*, 2000; Gorski *et al.*, 1994), our approach required a secondary clonotyping method for the quantification of frequency of individual selected clones. We developed a highly specific molecular tracking technique that allowed a direct *ex vivo* determination of clonal frequency without prior *in vitro* expansion. Our technique provided an improved sensitivity compared with previous methods, showing limits of detection ranging from 1 clonal cell in  $10^3$  irrelevant cells to 1 in  $2 \times 10^5$  (Moss *et al.*, 1995; Bieganowska *et al.*, 1997; Hanawa *et al.*, 1997; Pantaleo *et al.*, 1997; Nixon *et al.*, 1999; Babbe *et al.*, 2000; Kusaka *et al.*, 2000).



First, we asked whether the frequency of a T cell clone that recognized an immunodominant myelin epitope could be linked to markers of disease activity in a phasic disease such as RRMS. We tracked P2–10, a Th1 clone specific for MBP<sub>83–99</sub> that was possibly implicated in a disease exacerbation of a patient treated with an APL. The frequency of P2–10 increased strikingly after the start of APL treatment, reaching a peak of 1 cell in ~1800 PBMC just prior to the onset of a severe clinical exacerbation. This finding suggests a possible role of the clone as a trigger, rather than as a bystander, a secondary effector, or a regulatory cell population in this exacerbation. In fact, the kinetic of clone P2–10's frequency closely resembles that of encephalitogenic cells in animal models of multiple sclerosis. Indeed, autoimmune effector T cells that mediate EAE reach their highest frequency in the peripheral compartments before the onset of clinical disease and rapidly decrease when massive migration into the CNS is observed (Flugel *et al.*, 2001; Targoni *et al.*, 2001). To our knowledge, this is the first report of a human autoreactive Th1 clone reproducing in a patient with multiple sclerosis the kinetics of encephalitogenic T cells in EAE. An important implication is that an increased frequency of autoreactive effector T cells might only be detected during a short period of time preceding multiple sclerosis relapses. This notion may partly explain the conflicting results of studies comparing the frequency of myelin-reactive T cells in multiple sclerosis patients and controls. An additional important finding is the detection of P2–10 in the patient's peripheral blood over 6 years before the start of APL treatment. This observation, consistent with the results of a long-term follow-up of the MBP-specific T cell repertoire in multiple sclerosis patients (Goebels *et al.*, 2000), shows that the clone previously existed in the peripheral circulation and the APL stimulated its expansion. MBP-specific T cells can also be raised from the peripheral blood of healthy subjects (Ota *et al.*, 1990; Martin *et al.*, 1990; Pette *et al.*, 1990; Muraro *et al.*, 1997) and can persist over prolonged periods of time (Goebels *et al.*, 2000). Unlike the MBP-specific T cell clone from the multiple sclerosis patient, however, the frequency in the peripheral blood of the control clone 1C2 from the healthy donor remained stable for at least 4 years. These data support the concept that the dynamic instability with expansions and contractions of clonal frequency might be an important feature of a fraction of myelin-reactive T cells in RRMS (Muraro *et al.*, 2002). Our results do not show an involvement of T cell clone P2–10 in a second exacerbation that was associated with a moderately increased proliferative response to a PLP peptide, an observation reminiscent of surges of T cell reactivity to PLP<sub>184–209</sub> reported in multiple sclerosis patients, sometimes in association with clinical relapses (Pender *et al.*, 2000). The high frequency determined for clone P2–10 by our molecular tracking method supports the notion that function-based assays such as limiting dilution analysis may lead to an underestimate of the precursor frequency of autoreactive T cell clones, as previously suggested (Bieganowska *et al.*, 1997).

In HAM/TSP, we tracked T cell clone A6 which was isolated from a patient with a typical chronic-progressive disease course. A6 has been investigated extensively in previous studies, which provided remarkable insight on TCR structure and function (Garboczi *et al.*, 1996; Ding *et al.*, 1998, 1999; Baker *et al.*, 2000, 2001). Therefore, it was interesting to assess the possible pathogenic involvement of the clone by monitoring its frequency over time and in the CSF versus the peripheral circulation. We quantified the frequency of A6 in the peripheral blood as being on average 1 out of 850 PBMC. This frequency appeared

remarkably stable over a long period of time. In sorted CD8<sup>+</sup> T cells, clone A6 represented 1 out of 44 cells. Again, these frequencies indicate that limiting dilution analysis may underestimate the precursor frequency of *Tax*<sub>11-19</sub>-specific CTL in HAM-TSP patients, which had previously been calculated in the range of 1 out of 75 to 1 out of 320 cells in the CD8<sup>+</sup> subpopulation (1 out of 300 CD8<sup>+</sup> cells in the very same patient) (Elovaara *et al.*, 1993). A similar conclusion had been suggested by studies in which Ag-specific cells were enumerated using a *Tax*<sub>11-19</sub>-loaded HLA-A2 Ig fusion protein (Greten *et al.*, 1998) or a HLA-A2/*Tax*<sub>11-19</sub> tetramer (Bieganowska *et al.*, 1999). Our results showed that clone A6 alone represents 14.2%, or one out of seven, of all *Tax*<sub>11-19</sub>-specific, HLA-A2-restricted CD8<sup>+</sup> T cells in the peripheral blood. To our knowledge, this is the first direct measurement (i.e. without prior *in vitro* expansion) of the proportion of an individual T cell clone within the repertoire of T cells that recognize an MHC/Ag complex related to a neurological disease. The high degree of representation of this single clone suggests a possible involvement of the clone in the disease process, particularly in view of the degenerate TCR recognition of antigenic ligands (Mason, 1998). The most compelling support to this notion, however, comes from the fact that the frequency of T cell clone A6 in the CSF, determined by TCCT as being 1 out of 65 cells, is increased ~12-fold with respect to that in peripheral blood leukocytes.

In the patient with chronic Lyme neuroborreliosis, the enrichment of clone CSF3 in the CSF as compared with the peripheral blood was even more striking. In fact, despite the exquisite sensitivity of the primer/probe combination, no CSF3 clonotypic transcripts were amplified from any of six PBMC samples obtained from the patient over a period of 2.5 years. In contrast, CSF3 was consistently detectable in the CSF, and its frequency ranged from 1 out of 5555 to 1 out of 395 CSF cells, i.e. at least 900- to 12 000-fold greater than in the peripheral blood. The highest frequency was found at the time of a severe clinical exacerbation, while the lowest coincided with recovery, and an intermediate value was found during a mild exacerbation. The extreme bias of the frequency of CSF3 toward the site of inflammation and the correlation of clonal expansions with the clinical course supports the hypothesis of a pathogenic link between this clone and the disease process. We are in the process of extending these observations by examining matching PBMC and CSF samples collected during the most recent follow-up of the patient.

Taken together, our results suggest a relationship between the dynamics of frequency of functionally relevant T cell clones and the course of neurological disease. In a patient with RRMS we found that a putative pathogenic T cell clone that was previously detectable at lower frequency in the peripheral circulation became greatly expanded in close association with an inflammatory flare. In a patient with a chronic progressive disorder such as HAM/TSP, a candidate pathogenic clone had persistently high frequencies in the peripheral blood, was enriched several-fold in the CNS compartment, and accounted for a sizeable proportion of the overall T cell recognition of the target Ag/MHC complex. In the patient with Lyme neuroborreliosis, we found evidence of a substantial sequestration of autoreactive T cells within the target organ compartment. Our observations have implications for the pathogenesis of neurological immune disorders and may be important for designing successful therapeutic interventions. T cell clonotype tracking has great potential for further studies on immune-mediated diseases and in clinical trials.

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## Abbreviations

<b>APL</b>	altered peptide ligand
<b>CDR3</b>	complementarity-determining region 3
<b>C<sub>T</sub></b>	threshold cycle
<b>Flu-HA</b>	influenza haemagglutinin
<b>HAM/TSP</b>	HTLV-I associated myelopathy/tropical spastic paraparesis
<b>MBP</b>	myelin basic protein
<b>MRI</b>	magnetic resonance imaging
<b>PBMC</b>	peripheral blood mononuclear cells
<b>PLP</b>	proteolipid protein
<b>RRMS</b>	relapsing-remitting multiple sclerosis
<b>RT-PCR</b>	reverse transcriptase-PCR
<b>SEA</b>	staphylococcal enterotoxin A
<b>SEE</b>	staphylococcal enterotoxin E
<b>SI</b>	stimulation index
<b>TCCT</b>	T cell clonotype tracking
<b>TCR</b>	T cell receptor

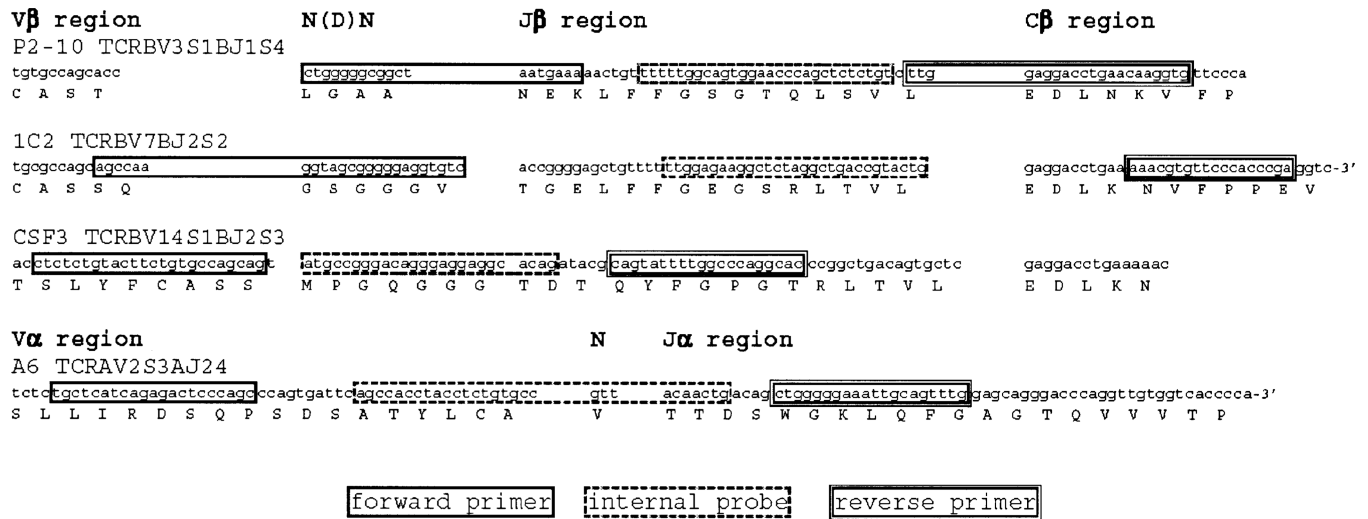
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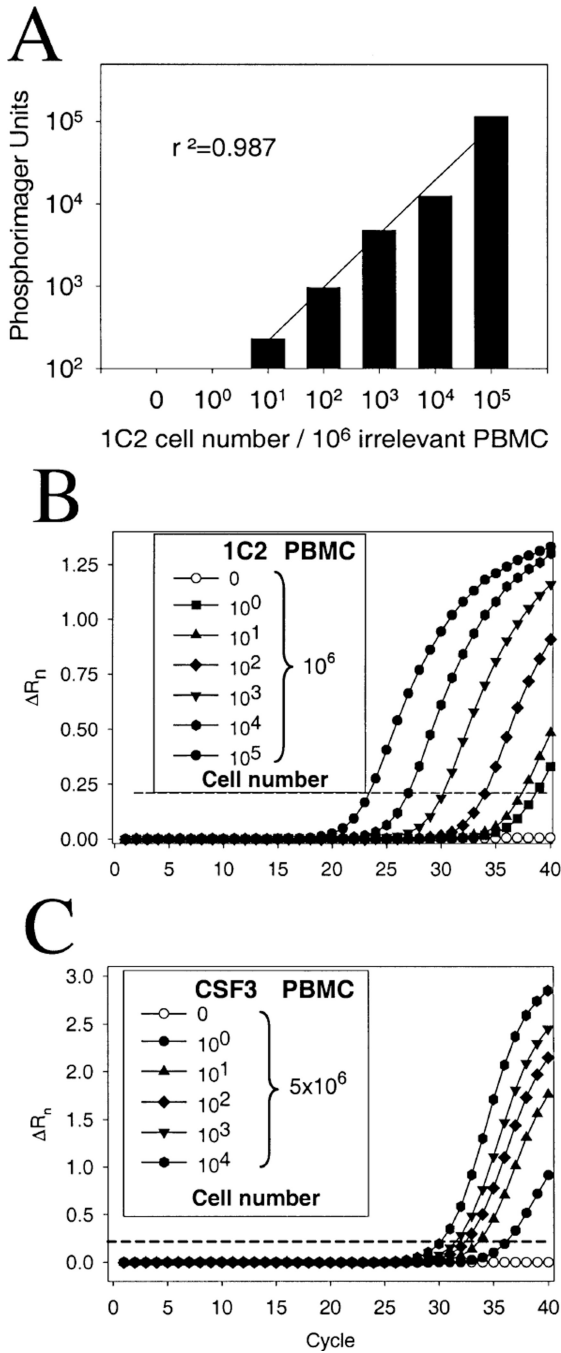
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**Fig. 1.**

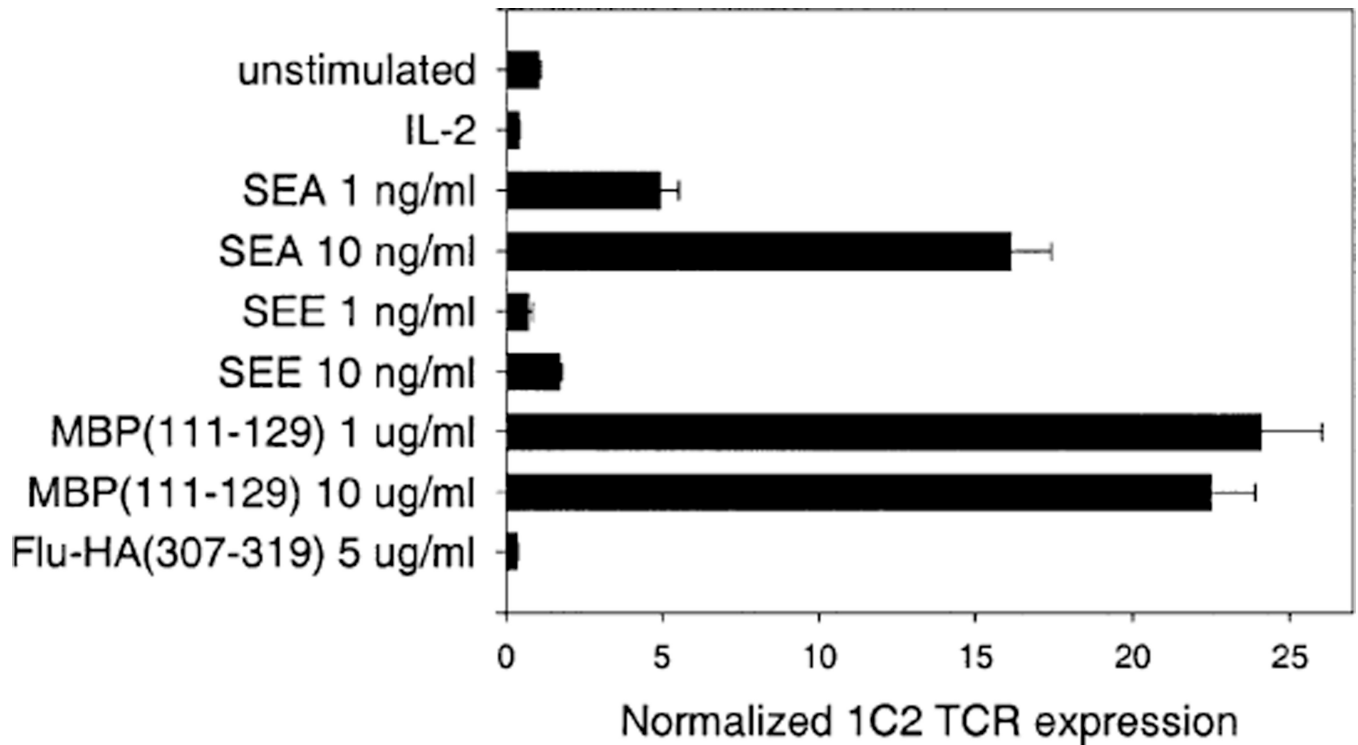
Annealing sites of clonotype-specific oligonucleotide primers and probes. The nucleotide and deduced amino acid sequences of TCR  $\beta$  (T cell clones, P2-10, 1C2, CSF3) or  $\alpha$  chain (T cell clone A6) CDR3 are shown. The annealing sites of oligonucleotide primers and probes for TaqMan quantification of clonotypic TCR transcripts from each T cell clone are indicated by boxes (single line, forward primer; dashed line, internal probe; double line, reverse primer).

**Fig. 2.**

High sensitivity of TCCT. ‘Spiking’ experiments assessed the sensitivity of TCCT for the detection of clonal cells at low frequency. **(A)** Conventional PCR and quantification of clonotypic TCR transcripts of T cell clone 1C2 using  $P_{33}$ -radiolabelled dCTP allowed the detection of 10 target cells/ $10^6$  irrelevant cells. **(B)** As compared with conventional PCR, TCCT by TaqMan real-time quantitative PCR yielded a 10-fold increase in sensitivity, resulting in positive amplification of a 1 : 20 dilution of cDNA derived from a mixture of 1 clonal cell/ $10^6$  control cells. **(C)** The outstanding efficiency of amplification of CSF3 TCR

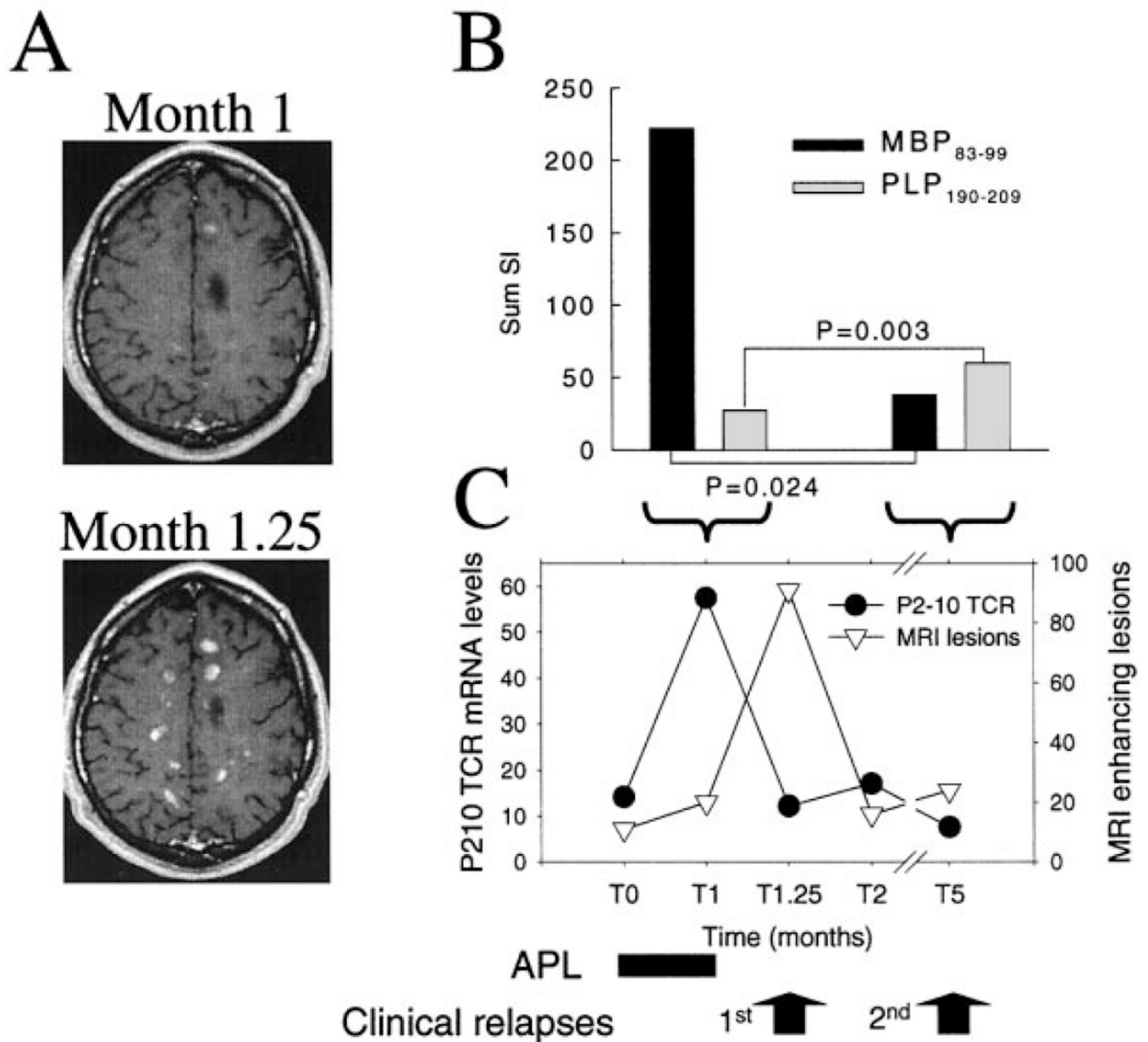


transcripts allowed detection of 1 clonal cell in  $5 \times 10^6$  control cells. High specificity of amplification was shown by the absence of any amplification of control cDNAs after up to 40 PCR cycles (Table 2).



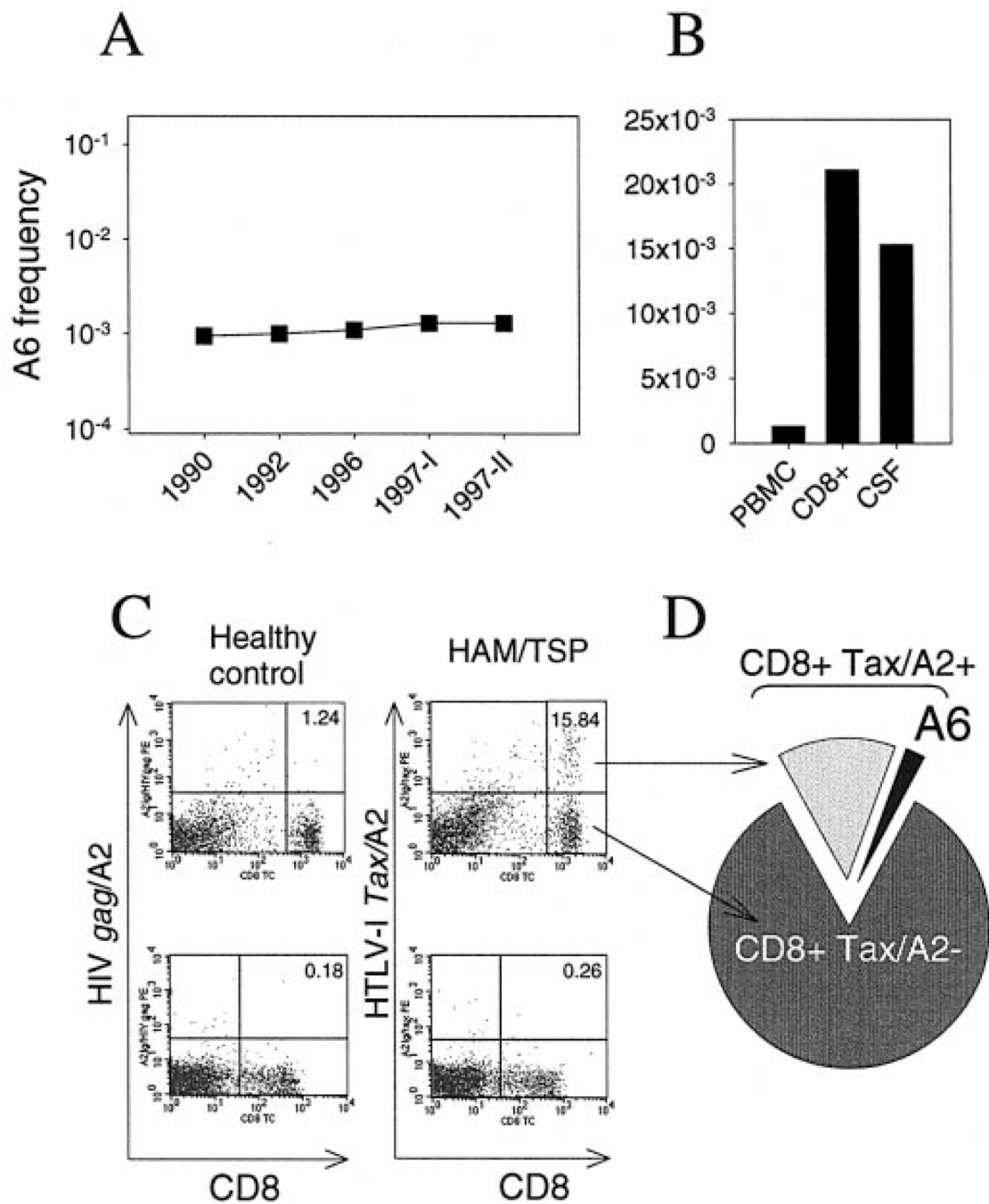
**Fig. 3.**

Detection of *in vitro* induced clonal expansion. Selective expansion of clone 1C2 in the unmanipulated 'parent' PBMC from donor HD4 was detected by TCCT as a result of stimulation with the specific antigen MBP<sub>111-129</sub> or with the superantigen SEA, which were known from previous experiments to stimulate proliferation of the isolated clone. There was no increase of 1C2 TCR transcripts upon PBMC stimulation with control stimuli FIU-HA<sub>307-319</sub> and SEE. Error bars indicate standard deviation.



**Fig. 4.** Expansion of MBP<sub>83-99</sub>-specific T cell clone P2-10 before a multiple sclerosis exacerbation. (A) Representative post-contrast T<sub>1</sub>-weighted brain MRI scans of patient MS502 at month 1 and at month 1.25 (exacerbation) of follow-up. (B) Cellular immune reactivity of PBMC from patient MS502 in response to the prevailing epitopes (MBP<sub>83-99</sub>, black fills; and PLP<sub>190-209</sub>, grey fills) being recognized at two different time-points (T1, left pair; T5, right pair), expressed as sum of stimulation indexes (SI) of all proliferating (SI > 2) wells in an IL-7-modified proliferation assay. *P* values were calculated using the Mann-Whitney rank sum test. A significant expansion of MBP<sub>83-99</sub>-specific T cells was observed at month 1 of treatment with APL. In contrast, at month 5 the response to PLP was increased. The lower variance of SI for PLP<sub>190-209</sub> accounts for the higher significance. (C) Molecular

quantification of P2–10 clonal frequency in PBMC (circles) over time (expressed in months; *x*-axis) is shown along with disease activity assessed by MRI (triangles). SD of P210 mRNA levels were: 1.5 (T0), 2.5 (T1), 1.2 (T1.25), 1.4 (T2) and 0.7 (T5). A 5-fold increase of P2–10 frequency (filled circles) was observed 1 week (T1) before the onset of a clinical exacerbation associated with the appearance of a large number of new gadolinium-enhancing lesions at MRI of the brain (open triangles). No increase of clone P2–10 was found at the time of a second relapse (T5).



**Fig. 5.** Persistent expansion of T cell clone A6 and relationship with CD8<sup>+</sup> T cell recognition of HTLV-I *Tax*<sub>11-19</sub>/A2 complex in a patient with HAM/TSP. (A) Stable frequency of A6 in the peripheral blood over several years of chronic progression of HAM/TSP. (B) Increased frequency of A6 in sorted CD8<sup>+</sup> cells and in unmanipulated CSF cells (12-fold). (C) Staining of PBMC from a healthy control (left) and patient HAM3 (right) with A2 Ig loaded with HTLV-I *Tax*<sub>11-19</sub> (top) or with a control peptide, HIV *gag*<sub>77-85</sub> (bottom). Percentages in upper right quadrants indicate the proportion of *Tax*/A2 Ig positive CD8<sup>+</sup> cells in the total

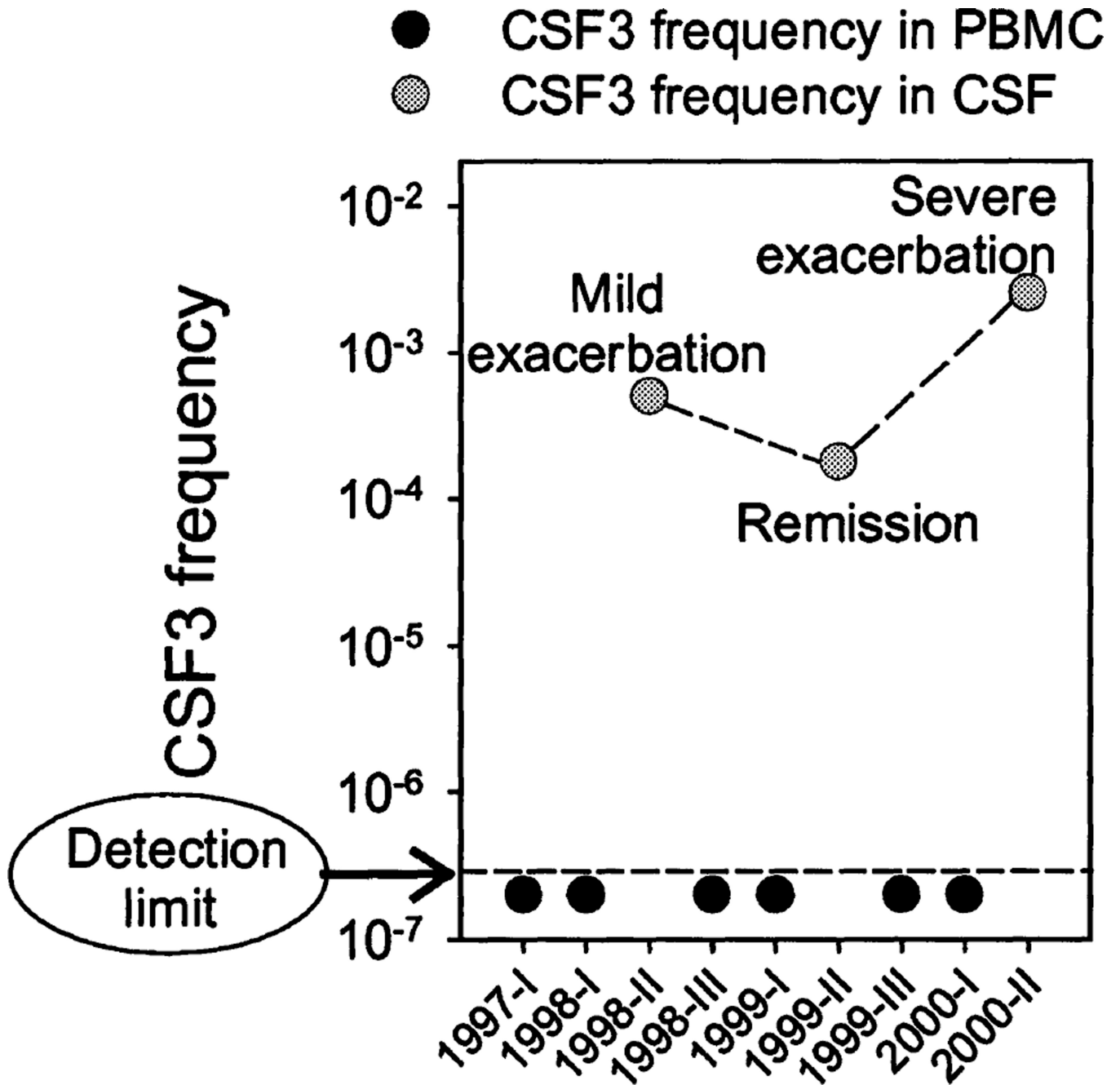
CD8<sup>+</sup> cells. **(D)** By staining with *Tax*/A2 Ig and TCCT, we determined that clone A6 alone represents 14.2% (or, one of seven) of all *Tax*<sub>11-19</sub>-specific, A2-restricted CD8<sup>+</sup> T cells in the peripheral blood. This figure can be viewed in colour as Supplementary material at *Brain* Online.

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**Fig. 6.** Frequency of T cell clone CSF3 in PBMC and CSF cells from a patient with Lyme neuroborreliosis. CSF3 TCR transcripts were undetectable in PBMC samples collected at 6 different time points. Lower limit of detection was 1 cell/ $5 \times 10^6$  (see Fig. 2C). In contrast, CSF3 was repeatedly found in CSF, with the highest frequency at the time of a severe exacerbation of neurological symptoms, an intermediate frequency during a milder relapse, and the lowest frequency at the time of clinical remission.

Table 1

T cell clones

T cell clone	Patient	Source	Specificity	HLA restriction	Phenotype	TCR $\alpha$ chain	TCR $\beta$ chain
P2-10	MS502	PBMC	MBP <sub>83-99</sub>	DRB1*0404	CD4 <sup>+</sup> Th1	ND	BV3S1U1S4
1C2	HD4*	PBMC	MBP <sub>111-129</sub>	DRB1*0401	CD4 <sup>+</sup> Th1 <sup>†</sup>	AV4S1AJ50	BV7S2BJ2S2
A6	HAM3	CD8 T cells	HTLV-I Tax <sub>11-19</sub>	A2	CD8 <sup>+</sup> CTL	AV2S3AJ24	BVA2S3DB2S1B12S7
CSF3	NB1	CSF	<i>B. burgdorferi</i>	DRB1*1501	CD4 <sup>+</sup> Th1	AV13S2AJ29	BV14B12S3

\* Healthy donor.

<sup>†</sup> Secretes substantial amounts of IL-10 (> ng/ml).



Table 2

Specificity of primers/probe sets

cDNA	Primers / Probe*			
	P2-10	1C2	A6	CSF3
P2-10	■			
1C2		▨		
A6			■	
CSF3				■
3A6				
MS18				
GP34				
GP14				
5F6				
GDBP				
5G7				
5A1				
PB1				
PB2				
PB3	▨	▨	▨	
PB4	▨	▨	▨	
PB5	▨		▨	
PB6				

■	mean $C_T = 0-20$
▨	mean $C_T = 21-25$
▩	mean $C_T = 26-30$
▪	mean $C_T = 31-35$
▫	mean $C_T = 36-39$
□	No amplification at 40 cycles

To test the specificity of each clonotypic primer/probe combination (columns) we amplified cDNA (rows) from target T cell clones (P2-10, 1C2, A6, CSF3;  $5 \times 10^3$  cells/reaction) and from controls that included additional non-target MBP-specific T cell clones (3A6, MS18, GP34, 5F6, GDBP, 5G7, 5A1) and allogeneic PBMC (PB1-PB6). Input cDNA was equivalent to  $5 \times 10^3$  cells/reaction for target clones and  $10^5$  cells/reaction for non-target controls.